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CULTURAL STUDIES OF A NEMATODE ASSOCIATED WITH PLANT DECAY

BY HAVEN METCALF

WITH ONE PLATE

It is well known that various nematodes are associated with plant disease; but aside from the gall-forming species, very few have been described from the standpoint of the plant pathologist. In descriptions of cases of root rot, joint rot, and " damping off," particularly such as are associated with fungi of the form-genus *Fusarium,* the presence of nematodes is frequently noted. The suspicion is often expressed that the nematode, rather than the fungus, may stand in a causal relation to the disease. But so far as I have been able to ascertain, no work has been done to demonstrate conclusively the relation of nematode to rot in any given case.

OCCURRENCE

My attention was first called to this species by Mr. J. L. Sheldon of the department of botany of the University of Nebraska, who noticed nematodes in great numbers in corms and young stalks of *Crocus,* which were affected with a soft rot; a *Fusarium* and bacteria were also present. In order to separate the fungus, poured plates were made with asparagus juice agar. To my surprise, not only the fungus and bacteria developed in the plate, but also, after about ten days, the nematodes. When first noticed, only three individuals were seen, but these multiplied until in about thirty days from the time of pouring the plate, the agar was fairly alive with nematodes, of all degrees of development. Trial inoculations of fresh tubes and plates showed that the nematodes could be grown readily by certain culture methods.

The same nematode was later isolated from cuttings of *Petunia, Coleus,* and *Geranium* which " damped off " in the green-house. In each case *Fusarium* was also found. In a number of sugar beets rotting with a characteristic bacterial rot nematodes were found

(Hedgcock and Metcalf, 1903); also in the late stages of the rot of potatoes caused by *Stysanus stemonitis* (Bessey, 1902). These potatoes were on sale in the markets of Lincoln, Nebraska, and were said to have been raised in Minnesota. The sugar beets were from Ames, Normal, and Grand Island, Nebraska. The bulbs and cuttings were from the green-houses of the University of Nebraska. Through the kindness of Mr. P. H. Rolfs, I have been able to examine certain *Coleus* plants from Miami, Florida, which were affected with some sort of root gall. From these galls no fungus nor animal parasite could be isolated, nor anything that would directly account for the abnormal growth. But about the galls considerable numbers of this nematode were found. Upon roots of the "iron pea" affected with a characteristic, but hitherto unstudied root rot, I have further found the same nematode in great numbers. The plants examined were from various points in Darlington, Orangeburg, and Oconee counties, South Carolina. The root rot in question is, in every case that I have observed, associated with a *Fusarium*, and the nematodes are always present. From these observations it seems probable that the nematode is widely distributed.

Structure and Classification

Only female forms have been observed in cultures or in decaying plant tissue. Culture experiments show that these female forms are sexually self-sufficient; isolated specimens develop from the egg, and produce eggs, which develop normally. No histological studies have been made, hence it is impossible to say with certainty whether the form is hermaphroditic or parthenogenetic. Observations of living worms and those stained *in toto* have not revealed the presence of spermatozoa.

The size of mature individuals is subject to considerable variation. The maximum length observed was 1.034 mm.; but specimens only 0.6 mm. have been observed with living larvae inside. Measurements of isolated specimens show that growth in length does not cease when egg production begins. The escape of larvae into the body cavity, however, results in the death of the parent. Detailed measurements of a mature individual of average size are as follows: length, 0.87 mm.; maximum width, 0.072 mm.; length of oesophagus, 0.19 mm.; length from anus to posterior extremity, 0.085 mm.; length of eggs, 0.036 mm.; width of eggs, 0.021 mm.

As the measurements show, the form is rather plump. From the middle toward the anterior end it tapers gradually, but from the anus to the posterior end rather abruptly, ending in a point. This portion is noticeably more attenuated and proportionally longer in the larva than in the adult. No rings or wrinkles are perceptible in the cuticula, which is perfectly transparent. The head end is blunt, with three lips, upon each of which is one very minute papilla (Figs. 3, 5). The buccal cavity is rod-shaped, of equal diameter at all points. Back of the buccal cavity the oesophagus is of nearly equal diameter for one third its length, then swells into an elongated bulb, which tapers gradually until it is scarcely larger than the buccal cavity. This portion then swells abruptly into the large globular bulb, which is supplied with a valvular apparatus. The intestinal wall is transparent in the adult, bright by transmitted light, with distinct cell boundaries and nuclei. At the point of juncture with the proctodaeum is a conspicuous cluster of large gland cells (Fig. 4).

The vulva is a trifle cephalad to the middle of the body (Fig. 3). The genitalia are very variable in extent and arrangement in different individuals; but approximately symmetrical. Sometimes the distal ends are reflected back towards the vulva; quite as often not. The posterior portion is most frequently reflected. Usually this does not extend for more than half the distance from vulva to anus; while the anterior portion usually extends to the bulb.

Development has not been studied, although no object could be more favorable for such study. Eggs are deposited in all stages of development, or if not deposited, the larvae develop in the egg, break out into the body cavity, where they continue to grow at the expense of the parent; ultimately breaking through the body wall. I have not been able to see that this process takes place at any particular period in the life of the parent. But sooner or later it seems to occur in every individual. Not always, however, does the escape of the larvae into the body cavity precede the death of the parent form; in a number of cases, the worm has died from some cause, and the escape of the larvae from the decaying genitalia and finally from the body occurred as a matter of course. Several worms containing eggs I have killed by mechanical means; in every case the fully formed eggs have continued to develop normally. Whether within or without the parent body, the larva may attain a length of

0.15 mm. before breaking out of the egg. At about this time the larva moults (Fig. 1); once again at about the time egg production begins.

While this form does not exactly correspond to any written description that I have seen, it is closely related to, if not identical with, the form described and figured by De Man (1884) under the name of *Rhabditis brevispina* Claus. The figures and measurements given by De Man agree substantially with mine; his description differs in certain particulars. According to him, " Das Kopfende . . . wird von drei, wenig hervorrugende Lippen gebildet, auf welchen sechs sehr wenig vorstehende Papillen gefunden werden "; I have observed only three papillae. The vulva is located in the middle or slightly cephalad of the middle of the body in all forms that I have examined; according to De Man, " Die weibl. Geschlechtsöffnung liegt ein wenig hinter der Mitte." I have seen no caudal papillae; but according to De Man, " Der Schwanz . . . trägt eine laterale Papillae ungefahr in seiner Mitte." Aside from these particulars De Man's description of the female applies perfectly to the form under consideration.

The original description by Claus (1862) of what he terms *Anguillula brevispinus,* is meagre; so far as it goes, his description of the female applies to the form which I have; and as papillae are not mentioned, it is in agreement with De Man's description. Regarding the position of the vulva, Claus says: " Die Geschlechtsöffnung liegt so ziemlich in der Mitte der Liebeslange."

Claus and De Man both describe male as well as female forms. Bütschli (1873) describes and figures a female form which he considers to be *Rhabditis brevispina* Claus. He says: " Der einzige bemerkenswerthe Unterschied, welchen ich auffand, ist, dass die Ovarien meiner Thiere bedeutend weiter nach vorn, respective nach hinten reichten, als dies von Claus angegeben." But between different individuals of the specimens which I have examined I have found greater differences in the arrangement and extent of the ovaries than is shown in the figures of Bütschli, Claus, and De Man. Regarding Bütschli's description De Man says: " Die, von Bütschli . . . als *brevispina* beschriebene Art ist eine andere und unterscheidet sich besonders durch die mehr beträchtliche Ausdehnung der Genitalien und einen verhältnissmässig kürzeren Schwanz." But a close comparison of the figures of the three authors shows that the tail of the female in De Man's figure differs as much in form from that depicted by Claus as that in Bütschli's figure does in length. Of the three figures, that of De Man most closely depicts the form that I have, in this as in other respects.

The form figured by Claus exhibits distinct differentiation of ovary, oviduct, uterus, and receptaculum seminis, such as is not shown in the figures of Bütschli and De Man. In the form that I have studied, there is no proper differentiation of ovary and uterus; the egg-producing and egg-retaining portions vary greatly in extent in different individuals (see Figs. 3 and 6).

Whether the forms described by the three authors are identical, and whether the form which I have described is identical with one or all of them, must be left for some other investigator to settle.

Cultures

My first cultures were made merely for following out the life history of the nematode; no attempt was made to keep the cultures pure. The original plates of asparagus agar, inoculated from rotting corms of *Crocus,* contained a *Fusarium* and one or more species of bacteria in addition to the nematodes. Culture slides of the ordinary pattern were prepared by placing distilled water in the slide, and a small quantity of agar on the cover glass. On each cover glass was placed a single worm or a single egg; and the cover glass was sealed to the slide with vaseline. On account of the large number of worms and eggs in the original culture, and their small size, it was not an easy matter to isolate an individual. It was accomplished by shaking a small portion of the culture in a few cubic centimeters of water in a small test-tube and pouring the whole out over the surface of a Petri dish. The worms and eggs, separated in this way, could be easily located with a lens, and picked up with a brush without injury.

Fungi and bacteria developed in the cultures. In the decaying mass the nematodes grew rapidly, and so far as could be judged by comparing with specimens in the rotten plant tissue, normally. The limited air supply seemed to cause no difficulty; at least no difference could be detected in the behavior of worms in these culture slides and in aerated Van Tieghem cells or in Petri dishes. The following are notes taken upon a typical preparation:

March 1, 1902. One egg, unsegmented, placed in a culture slide.

“ 2. Egg unchanged.

“ 3. Egg unchanged.

“ 4. Egg segmented, apparently fourteen cells.

“ 5. Egg further segmented.

“ 9. Embryo distinctly formed.

“ 11. Embryo moving about actively in egg.

“ 12. Embryo broken out of egg.

“ 13. Embryo moulting; proton of genitalia visible; worm 0.18 mm. long.

“ 14. Moulting complete.

“ 17. Proton of genitalia 0.032 mm. long, of female type (Fig. 2, a); worm 0.34 mm. long.

“ 20. Worm 0.56 mm. long; genitalia 0.15 mm. long, recurved at anterior end. Skin loose at both extremities.

“ 22. Second moulting complete; worm 0.56 mm. long.

“ 24. Three unsegmented eggs formed; worm 0.65 mm. long.

“ 25. Three eggs segmenting; six others formed.

“ 26. Four eggs deposited; worm 0.73 mm. long.

“ 27. Three more eggs deposited.

“ 28. Two eggs in anterior branch of genitalia segmenting.

“ 30. Eggs further segmented.

April 2. Embryos in eggs fully formed and active; eggs still within the genitalia.

“ 3. Embryos in eggs moving actively.

“ 4. Larvae broken through eggs and wall of genitalia, moving actively in posterior part of body cavity; parent worm not moving about, but alive and feeding as shown by characteristic motions of valvular apparatus of bulb. One larva measures about 0.15 mm. in length.

“ 5. Parent worm alive.

“ 6. Parent worm apparently dead; genitalia decaying; both larvae moulting.

“ 9. Both larvae still inside body of parent worm, of which only chitinous portions remain; both larvae have moulted; one measures 0.38 mm. in length; its genitalia extend one-sixth the length of the body.

April 14. Both larvae escaped from skin of parent worm; the seven eggs deposited in the medium have developed; there are now nine worms in the culture; largest worm measures about 0.62 mm.; three worms have one or more eggs developing; preparation moist, but worms not moving very actively.
" 15. Worms not moving or feeding; several coiled up.
" 18. All worms more or less coiled; no motion.
" 20. Condition unchanged.

Nothing further was done with this preparation. In another preparation where quiescence ensued in the same way, after forty-two days from the time the preparation was sealed, it was noticed that the worms were not dead,—at least they were not attacked by bacteria on the fifty-sixth day. Accordingly the preparation was opened and the worms transferred to a fresh Petri dish, where they revived and continued to develop and produce eggs. This quiescence has no relation to air supply, since after a certain time it ensued in Petri dishes and other aerated cultures. It is not improbably due to accumulation of waste products, *e. g.*, urea. Apparently nothing but new medium will revive the worms. This is probably to be correlated with the fact that the worms naturally move about over a large area, continually seeking a new substratum.

Up to the time that this quiescence begins to appear, the conditions in the cultures appear to be entirely normal. I have made elaborate comparisons of the nematodes in cultures with those growing under perfectly normal conditions in decaying plant tissue; apparently there is not a stage or condition occurring in cultures that cannot be matched, specimen for specimen, among those living naturally.

Methods of observation

The living worm is in constant motion, and is consequently difficult to observe with high powers of the microscope. On the other hand the living worm is transparent, while most killing media soon render some tissues opaque. Narcotizing the worms naturally suggests itself as a possible method of keeping the worms quiet for observation while retaining the transparency of life. With a 0.1 per cent solution of chloral hydrate and with a 1 per cent solution of cocaine hydrochlorate I had fair success; the only objection being the slowness of action: worms treated with chloral do not entirely cease motion for thirty minutes. The most practicable method of preparing worms for observation was by treating them with a 0.01 per cent solution of mercuric chloride; death was practically instantaneous, and the worms did not begin to lose transparency for from thirty to forty-five minutes; allowing time for drawing and observations.

Obtaining sterile nematodes

In order to make inoculations upon living plants to determine whether the nematodes have any pathogenic power, it was first necessary to secure worms free from bacteria, fungi, or any other organisms. This proved to be by no means an easy matter. I first tried to free eggs from bacteria and fungus spores by making plates from them in the usual way, with asparagus agar. While by this process the germs were scattered, some bacteria or fungus spores remained so near the eggs that the latter could not be absolutely isolated. Various methods of sterilizing the eggs by chemical means were then tried: eggs were washed for varying lengths of time in various solutions of mercuric chloride, carbolic acid, thymol, copper sulphate, dilute hydrochloric acid; with the uniform result that whatever destroyed the plant organisms destroyed the eggs also. I then hit upon the method of washing eggs in sterile water, placing them in a watch glass, and changing the water repeatedly with a pipette. The eggs sink in water; so also do most bacteria, but spores of *Fusarium* and of terrestrial fungi in general float. Hence by this method the eggs were easily freed from fungus spores, but not from bacteria. But by repeated washings the surface of the eggs was largely freed from bacteria, and the number of bacteria in the water greatly reduced. Then the eggs were placed in liquefied agar tubes at low temperature, and poured plates made in the usual way. In the plates several spots of fungi appeared, and many bacterial colonies. But out of the twenty eggs used five were so situated that after two days' growth at room temperature no colonies were near enough to touch them. These eggs were then transferred to plates of sterile agar by using a special form of flat oese, a description of which will shortly be published. In this way eggs were secured free from all micro-organisms.

The sterile agar into which the eggs were transferred was a one per cent asparagus juice agar, sufficiently moist, but rather stiff. In this medium the eggs developed, the larvae moulted, but after the first moult made very little growth. At first they moved about freely, but after from ten to fifteen days they curled up and became quiescent. That they were free from moulds and bacteria was conclusively shown by the fact that the agar remained uncontaminated.

As I was uncertain why the worms failed to develop, and was inclined to attribute the difficulty to injury received in the washing, I repeated the experiment twice, with similar results. It then occurred to me that the difficulty might be with the medium: that products of decay might be necessary food. Accordingly I inoculated a flask of one per cent asparagus juice agar with the *Fusarium* and bacteria of one of the original poured plates; after allowing the mass to decay for two weeks I heated the agar, filtered out the fungus fiber, and sterilized the filtrate. Sterile eggs were then placed in sterile plates of this agar; they developed rapidly and normally, and the worms produced eggs in their turn.

This decayed agar, however, was more nearly liquid than the normal agar. In order to show whether degree of solidity might not have as much to do with the behavior of the nematodes as presence of decomposition products, I placed other sterile eggs in a 0.25 per cent asparagus juice agar. These developed, not as rapidly nor as vigorously as those in the decayed agar; but quiescence did not ensue until several generations had developed. But as decayed agar was distinctly the best medium, it was used wholly in growing sterile worms for inoculation purposes.

I have already mentioned that these nematodes move about actively, and over considerable area if given range. Their food also passes quickly through the alimentary tract; it occurred to me that these facts might be utilized in devising a method of freeing the living worm from micro-organisms. To this end several rectangular Petri dishes were constructed, measuring three by fifteen centimeters; in these 0.25 per cent asparagus agar was placed; after this was sterilized, the dishes were slanted, so that the water collected largely at one end. Active worms were then placed in the upper end; they immediately began to move toward the moist end, reaching it in a few hours; when they were transferred to the next dish and the process repeated. It was expected that this continual passage through sterile medium would free the worms, inside and out, of germs. But such did not prove to be the case. Although the number of organisms in and about each worm was greatly reduced, as was shown by the number of colonies developing along the trail, no worms could be obtained entirely free from either bacteria or fungi. This suggested what later experiments have demonstrated, that these nematodes are efficient agents in disseminating micro-organisms.

Biochemical Relations

This nematode when grown in cultures exhibits peculiar and interesting relations to its substratum, which I have not worked out. The medium becomes more alkaline, probably in consequence of the considerable quantity that passes through the worms.

The frequent occurrence of the nematodes with *Fusarium* suggests some vital relation of that fungus or its products; whether or not there is such relation, it is certain that some other fungi exert a deleterious effect upon the nematodes. I noticed that the nematodes died in a culture which had become contaminated with a black *Aspergillus.* I inoculated two other plates of worms with this fungus with the same result; the bad effect of the fungus growth was unmistakable. No investigation was made of the by-products of this *Aspergillus,* which are probably poison. Might not the line of investigation here suggested be fruitful if followed out with reference to certain pathogenic nematodes? Nothing is known, for example, regarding the relation of gall-forming nematodes to plants other than their hosts.

To dryness the live nematodes are fairly resistant, but not so much so as might be expected; in agar cultures dried at room temperature for twenty-four days the nematodes have failed to revive. The eggs do not seem to be much more resistant than the living animal, a fact which may be correlated with their frequent internal development.

The nematodes are unaffected by sunlight. No attempt has been made to work out their relation to salts, disinfectants, or other chemical substances.

INOCULATIONS

Inoculations of pure cultures of nematodes have been made upon young *Coleus* and *Geranium* plants, upon sugar beets, and upon the iron pea. The standard methods of inoculation were employed, and each inoculation carefully checked; so the methods need not be described in detail. Suffice it to say that wounds were made on parts of the plants under ground, and the nematodes placed on these spots. The wounds were kept moist. Although in every case the worms, or at least some of them, lived, decay of the plant tissue did not ensue in any case; instead, the wounds healed normally. Evidently, then, the nematodes alone have no pathogenic power.

More interesting results were obtained, however, upon using nematodes grown in pure cultures of the bacterium which causes the rot of sugar beets, already referred to. Some of these were placed on a wound in a live beet, with the prompt result of the decay of the beet by the bacterium. The experiment was also made of putting a quantity of nematodes from the same source on the surface of the soil around four potted beets. No decay ensued. But when the experiment was modified by wounding the surface of the beet *under the soil,* decay ensued in three out of four beets. The experiment was repeated with two other beets; both decayed; and examination of the decaying spots showed the nematodes to be present on the surface.

Cuttings of *Coleus* were placed in a pot of earth, and a large number of nematodes from a culture obtained originally from a *Coleus* cutting which had " damped off," were placed in the soil. *Fusarium* was present in the culture. Examination of the cuttings in ten hours showed that the nematodes had congregated around the cut ends of the plants. Later about one third of the plants " damped off." This led me to examine again fresh wounds of sugar beets, near which nematodes had been placed. Without exception the results showed that the nematodes gather about wounds; probably for the plant juices, upon which they seem to feed. Herein, then, lies their real relation to plant decay: they are carriers of germs of decay to wounded places. They are, however, necessarily from their structure, incapable of themselves producing the wounds.

SUMMARY

1. A nematode, *Rhabditis brevispina* (Claus) Bütschli or a closely related form, is commonly and widely associated with decay in certain plants.

2. The nematodes grow readily in agar cultures of plant juice if sufficiently fluid; better in decayed media. So far as can be determined by microscopical examination, the nematodes grown in cul-

tures are similar in all respects to those living under absolutely natural conditions.

3. Absolutely sterile cultures of the nematodes can be obtained by washing the eggs, and afterward making poured plates with them in the usual way.

4. In cultures the nematodes are killed by the presence of a certain species of *Aspergillus*.

5. The nematodes seek wounded places on the underground parts of certain plants, probably in order to feed upon the plant juices. If they bear spores of pathogenic organisms they necessarily inoculate the plants; and as they feed on decaying plant tissue, becoming covered with the germs of the decay, they readily transfer the disease from plant to plant.

ACKNOWLEDGMENTS

These studies have for the most part been carried on in the zoological laboratory of the University of Nebraska, and under the direction of Dr. Henry B. Ward, to whom I acknowledge many obligations.

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EXPLANATION OF PLATE VII

All figures drawn with Abbé camera lucida; 1, 3, and 6, from narcotized specimens, the remainder from specimens freshly treated with mercuric chloride.

Fig. 1. Larva at time of first moult. *a*, proton of reproductive organs.

Fig. 2. Portion of nematode from a culture sixteen days old. *a*, reproductive organs.

Fig. 3. Nematode from culture twenty-six days old.

Fig. 4. Posterior end of adult nematode. *a*, gland cells.

Fig. 5. Anterior end of the same specimen.

Fig. 6. Portion of middle of nematode from a culture twenty-two days old, showing posterior branch of reproductive organs.

PLATE VII

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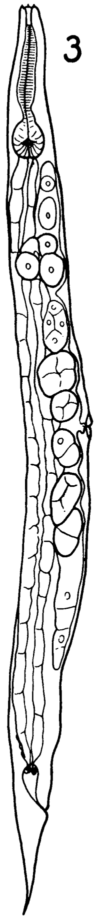

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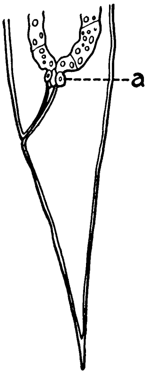

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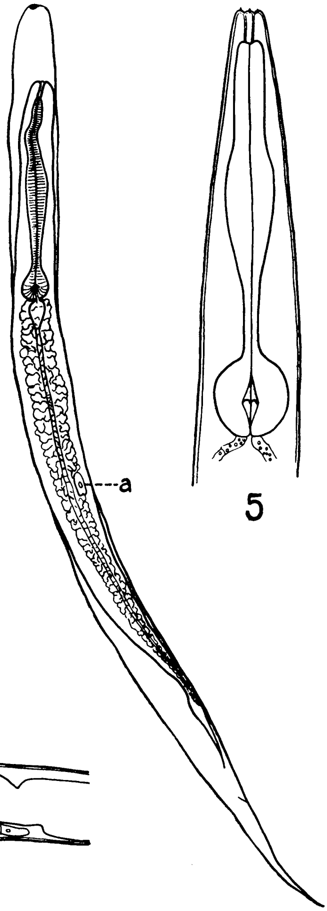

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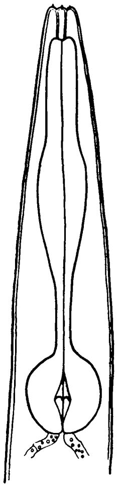

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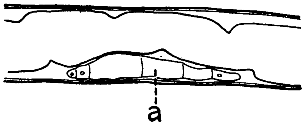

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